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Potent Oncolytic Activity of Multimutated Herpes Simplex Virus G207 in Combination with Vincristine against Human Rhabdomyosarcoma[1]

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ABSTRACT

Replication restricted oncolytic viruses such as multimutated herpes simplex virus type 1 (HSV-1) G207 represent a novel and attractive approach for cancer therapy, including pediatric solid tumors. Rhabdomyosarcoma is the most common soft-tissue sarcoma of childhood and is often diagnosed already as an advanced disseminated disease. Despite aggressive therapeutic approaches, the prognosis for patients with metastatic rhabdomyosarcoma remains grim. Therefore, there is a need for novel effective drugs with superior safety and efficacy profile. In this study, we showed marked *in vitro* activity of HSV-1 G207 against embryonal and alveolar rhabdomyosarcoma cells. All human embryonal (KF-RMS-1, RD, and CCA) and alveolar RMS (KFR, Rh28, Rh30, and Rh41) cell lines were highly sensitive to cytotoxic and replicative effects of G207 even at a multiplicity of infection of 0.01, except embryonal Rh1 rhabdomyosarcoma cells, which were efficiently killed only upon multiplicity of infection of 1.0. i.v. G207 treatment of xenotransplanted KFR and KF-RMS-1 tumors in mice led to significant tumor growth inhibition of both tumor entities, whereas intraneoplastic G207 treatment additionally resulted in complete tumor disappearance in 25% of animals. No difference has been found between alveolar and embryonal types of rhabdomyosarcoma. Combination treatment of both cell lines with G207 and vincristine led to strongly enhanced *in vitro* cytotoxicity without affecting infection efficiency and replication of G207 in KFR as well as in KF-RMS-1 cells. *In vivo* combination treatment using i.v. G207 and vincristine resulted in complete regression of alveolar rhabdomyosarcoma in five of eight animals and significant growth inhibition of embryonal rhabdomyosarcoma. Taking into consideration the proven safety of G207 in humans, we suggest that G207 alone and in combination with vincristine should be additionally evaluated as a potential agent against human rhabdomyosarcoma.

INTRODUCTION

Rhabdomyosarcoma is the most common soft-tissue sarcoma of childhood accounting for more than half of this type of tumor and for 4–8% of all cases of malignant disease in childhood (1, 2). Rhabdomyosarcoma is composed of two major categories, those displaying embryonal or alveolar histology and presenting with distinct clinical, molecular, and genetic features. ERMS[3] is characterized by loss of heterozygosity at the 11p15 locus and usually occurs in younger patients, more frequently in head and neck region. In contrast, ARMS is diagnosed predominantly in older children who clinically fare worse. It most commonly affects extremities and harbors a specific chromosomal marker t(2;13)(q35;q14; Refs. 3–6). About 20% of children with rhabdomyosarcoma present with disseminated disease at diagnosis (7). Current treatment protocols include conventional chemotherapy, megatherapy, surgery, and irradiation. However, because of insufficient local control, the prognosis remains grim (5, 6, 8–10). In addition, the narrow therapeutic index of chemotherapy and irradiation demands new highly selective strategies to improve survival without exceedingly compromising quality of life.

Viruses used for oncolytic therapy are restricted in their ability to replicate to transformed cells, killing them through a direct cytopathic effect and enabling the viral progeny to spread within the tumor, sparing therefore nontransformed surrounding cells (11). HSV-1 G207 belongs to a second generation of genetically engineered HSV-1 mutants and has been clinically tested in a Phase I study in brain tumor patients (12). It harbors deletions in both copies of the *γ34.5* gene, the major determinant of HSV-1 neurovirulence (13, 14), and carries an insertion of the *Escherichia coli lacZ* gene in the viral *ICP6* gene (UL39), inactivating RR (15). Because viral replication can only take place in the presence of RR, replication of G207 is limited to dividing cells expressing high levels of RR (16, 17). Insertion of a *lacZ* marker gene, which produces a histochemically identifiable protein product, enables easy identification of viral infection (18). The multiple mutations of G207 minimize the chance of reversion to wild-type virus and confer additional safety features such as sensitivity to ganciclovir and temperature sensitivity, which halts viral activity in the febrile host (18, 19).

Experimental studies demonstrated that G207 is effective against human carcinoma cell lines of breast (20), prostate (21), colon (22), the ovaries (23), and head and neck SCC (24), malignant melanoma (25), as well as pediatric embryonal tumor cell lines such as neuroblastoma (26, 27). Two studies demonstrated that the combination of chemotherapeutic drugs with replication-competent HSV vectors augmented efficacy (24, 28). One investigation showed that the antitumoral activity of G207 after intraneoplastic inoculation of human head and neck SCC was significantly enhanced by additional treatment with cisplatin (24). The other study demonstrated that HSV-1 1716, containing deletions in both *γ34.5* alleles (parental HSV-1 strain 17^+), had at least additive or even synergistic antitumoral effects in various human non-small cell lung cancer cell lines when combined with mitomycin C (28).

In this study, we investigated and compared the oncolytic activity of G207 to several ARMS and ERMS cell lines. Because vincristine plays a critical role in rhabdomyosarcoma therapy, we investigated if the combination of G207 with vincristine would be more beneficial for the treatment of rhabdomyosarcoma tumors *in vitro* and *in vivo*. Because antimicrotubule agents, including vincristine, were shown to delay transport of HSV capsids to the nucleus of infected cells (29), we tested whether vincristine at therapeutic levels influences replication and antitumoral effects of oncolytic G207.

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[3] The abbreviations used are: ERMS, embryonal rhabdomyosarcoma; ARMS, alveolar rhabdomyosarcoma; HSV, herpes simplex virus; HSV-1, HSV type 1; RR, ribonucleotide reductase; SSC, squamous cell carcinoma; MEM, minimal essential medium; MOI, multiplicity of infection; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; PFU, plaque forming units; i.t., intratumorally.

MATERIALS AND METHODS

Virus. G207 was kindly provided by NeuroVir (Vancouver, British Columbia, Canada). Viral stocks were prepared by infecting African green monkey kidney cells (Vero; American Type Culture Collection, Manassas, VA) cultured in MEM supplemented with 5% inactivated FCS at a MOI of 0.01 at 34°C and harvesting virus when complete cytopathic effect was observed. After a freeze-thaw/sonication regime, cell debris was removed by low-speed centrifugation (2000 × *g* for 10 min at 4°C). The virus was concentrated by subsequent high-speed centrifugation (45,000 × *g* for 150 min at 4°C). The viral pellet was then resuspended in 150 mM NaCl and 20 mM Tris (pH 7.5). Infectious titers of viral stocks were determined by plaque titration on Vero cell monolayers as previously described (30) and stored at −80°C before use.

To investigate whether antitumor effects of G207 were attributed to viral replication, inactivated G207 was used as a control. Inactivation of virus was achieved by exposure of viral solution to UV-B light (280–350 nm, peak at 306 nm) for 25 min delivering ~3.6 J/cm^2. UV-irradiated virus suspensions were free of infectious virus as demonstrated by plaque titration using Vero cell monolayers.

Cell Lines. Human rhabdomyosarcoma cell line RD (embryonal subtype) was obtained from American Type Culture Collection. Rh1 (embryonal subtype), Rh28, Rh30, and Rh41 (alveolar subtypes) were kindly provided by Dr. Peter J. Houghton (St. Jude's Children's Research Hospital, Memphis, TN). CCA cell line (embryonal subtype) was a gift from Dr. Pier-Luigi Lollini (Department of Experimental Pathology, University of Bologna, Italy). KF-RMS-1 (embryonal subtype) and KFR (alveolar subtype) were established in our laboratory from bone marrow metastases of two of our patients suffering from ERMS and ARMS, respectively. Characteristics of cell lines including RD, Rh1, Rh28, Rh30, Rh41, CCA, and KFR, have been described previously (31–37). Cells were maintained in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator. Cells were routinely tested for *Mycoplasma* and found to be free of contamination.

Oncolysis of Human Rhabdomyosarcoma Cells. For *in vitro* susceptibility assays, rhabdomyosarcoma cell lines were plated in 6-well dishes at a density of 2 × 10^4 cells/cm^2. After 48–72 h, subconfluent cell layers were infected with G207 at MOIs of 0.01, 0.1, 1.0, or mock infected with viral buffer alone. After adsorption period (90 min) at 37°C, infected cells were maintained in growth medium containing 1% heat-inactivated fetal bovine serum at 37°C. Viable cells were counted in a hemocytometer using trypan blue exclusion at days 1–6 after infection. In experiments assessing the combination of G207 and vincristine, KFR or KF-RMS-1 cells were incubated with different concentrations of vincristine, which were added to culture medium immediately after the viral adsorption period.

Infection Efficiency and Viral Growth of HSV-1 G207 in Tumor Cells. To evaluate infection efficiency of G207 in different rhabdomyosarcoma, X-gal (Sigma, Taufkirchen, Germany) staining was performed using a previously described technique (38). The percentage of *lac*Z-positive cells was calculated as a measure of infection 4 days after infection. To assess replication capacity of virus in susceptible cells, viral titers were measured in the KFR cell line. Subconfluent layers of KFR cells were incubated with G207 at a MOI of 0.1 for an adsorption period (90 min), washed three times with PBS, and culture medium with different concentrations of vincristine was added. Immediately after virus adsorption (input virus titer) or 4 days after infection, cultures were subjected to three cycles of freeze-thaw lysis, sonication, and centrifugation at 2000 × *g* for 10 min at 4°C. Infectious titers of supernatants were determined on confluent Vero cell layers as described above.

s.c. Xenotransplanted Tumor Model of Human Rhabdomyosarcoma. Female outbred athymic nude mice, strain CD-1 (nu/nu), ~20 g of weight (AnLab Ltd., Charles River, Czech Republic) were used for experiments. Mice were kept under sterile conditions, receiving sterile nutrition and water. A total of 1 × 10^7 human rhabdomyosarcoma cells (KFR or KF-RMS-1) was injected s.c. together with Matrigel (Collaborative Biochemical Products, Bedford, MA) in a total volume of 0.2 ml into the right flank of mice. Tumor volumes were determined using a caliper and calculated by the formula: volume = (length × width^2)/2. The longer side was defined to be the length and the shorter one to be the width.

Intraneoplastic Treatment of Human Rhabdomyosarcoma Tumors with HSV-1 G207. Xenotransplanted KFR and KF-RMS-1 tumors were established as described above. After tumor cell inoculation, mice were randomly divided into groups of eight animals. When tumors reached a size of ~100 mm^3, treatment was started. This day was defined to be day 0. Animals received 1 × 10^7 PFU G207 i.t., suspended in a volume of 20 μl of virus buffer at days 0 and 4. Control animals received i.t virus buffer or UV-irradiated G207 virus suspension.

i.v. Treatment of Human Rhabdomyosarcoma Tumors with HSV-1 G207. Xenotransplanted KFR and KF-RMS-1 tumors were established as described above. Mice were randomly divided into groups of eight animals after tumor cell inoculation. Treatment was started when tumors reached a size of ~100 mm^3. This day was defined to be day 0. One group of animals received 2 × 10^7 PFU G207 i.v. into the tail vein, suspended in a volume of 100 μl of virus buffer at days 0 and 4. The next two groups were treated with either vincristine i.v. 0.5 mg/kg body weight at day 1 or vincristine in combination with G207. Control animals received viral buffer or UV-irradiated G207 viral suspension.

***In Vivo* G207 Dissemination Studies.** To test whether viral infection spreads over time in tumors treated with i.v. G207 or by combination of i.v. vincristine and G207, X-gal staining after single i.v. injection of 2 × 10^7 PFU of G207 was performed in tumors excised on days 1 and 5. Tumors were rinsed in PBS, fixed with ethanol, and cut into 5-μm slices. X-Gal staining was performed as described above and counterstained with eosine solution.

Electron Microscopy. For ultrastructural investigations of viral infection, mice bearing rhabdomyosarcoma tumors treated systemically with G207 or with the combination of G207 + vincristine were sacrificed on day 5 of treatment. Tumor specimens were fixed in 4% formalin and 1% glutaraldehyde in monobasic phosphate buffer (pH 7.2), postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Durcupan Fluka (Sigma). Thin sections were contrasted with uranyl acetate and lead citrate and viewed with a Jeol JEM, 2000 CX microscope (Arishima, Japan).

Statistics. Statistical analysis was performed using Jandel SigmaStat 2.0 (Jandel Scientific, Erkrath, Germany). For comparison of groups, Student's *t* test was used. Data groups were considered significantly different when $P < 0.05$.

RESULTS

Susceptibility of Human Rhabdomyosarcoma Cell Lines to HSV-1 G207 *in Vitro*. The oncolytic activity of G207 was assessed *in vitro* on eight human rhabdomyosarcoma cell lines derived from either ERMS (KF-RMS-1, RD, CCA, and Rh1) or ARMS (KFR, Rh28, Rh30, and Rh41). G207 exerted a direct dose- and time-dependent cytopathic effect on all rhabdomyosarcoma cell lines except Rh1 (Fig. 1). ERMS (KF-RMS-1, RD, and CCA) and ARMS (KFR, Rh28, Rh30, and Rh41) were highly sensitive to G207 with 100% cell death in cultures infected at a MOI of 1 and at least 85% of cells killed even at a MOI of 0.01 within 6 days. The ERMS Rh1 cell line showed a lower sensitivity to G207, *i.e.*, G207 had no effects on the number of viable cells in cultures infected at a MOI of 0.01. Fifteen percent of viable cells were found after 6 days in cultures infected at a MOI of 1. The oncolytic activity of G207 correlated inversely with confluency of cell layers (data not shown).

Combination Treatment of Human Rhabdomyosarcoma Cells with G207 and Vincristine *in Vitro*. To test whether vincristine influences cytotoxic activity of G207 against human rhabdomyosarcoma cells, KFR or KF-RMS-1 cultures were infected with virus at a MOI of 0.1 and incubated for 4 days with or without vincristine at concentrations ranging from 0.1 to 0.4 ng/ml. In cultures treated with the combination of G207 and vincristine at a concentration of 0.1 ng/ml, vincristine led to significantly enhanced cell killing when compared with respective single treatments. As shown in Table 1, KFR cells were all dead after incubation for 4 days with a combination of G207 and vincristine at a concentration of 0.2 or 0.4 ng/ml, whereas at least 30% of viable cells were counted in cultures treated

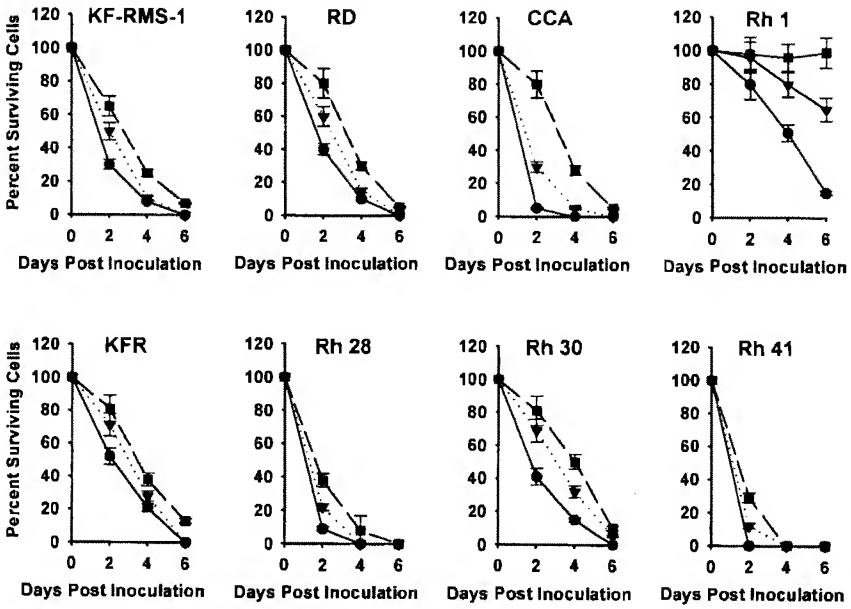

Fig. 1. G207 susceptibility of human rhabdomyosarcoma cell lines *in vitro*. Four embryonal rhabdomyosarcoma cell lines (KF-RMS-1, RD, CCA, and Rh 1) and four alveolar rhabdomyosarcoma (KFR, Rh28, Rh30, and Rh41) were infected at MOIs of 1 (●), 0.1 (▼), and 0.01 (■). Each data point (mean of triplicate wells ± SD) is the percentage of surviving cells compared with number of mock-infected cells in control wells at each time point.

Table 1 *Effect of vincristine on G207 mediated killing of KFR and KF-RMS-1 cells in vitro*

Treatment	% of survived cells	
	KFR	KF-RMS-1
vincristine (ng/ml)		
0.1	78.1 ± 18.2[a]	76.2 ± 13.9
0.2	49.8 ± 12.4	54.5 ± 12.8
0.4	39.6 ± 8.6	47.2 ± 14.2
G207 (MOI 0.1) + vincristine (ng/ml)		
0	37.2 ± 10.5	46.1 ± 12.3
0.1	24.7 ± 7.1[b]	37.2 ± 9.6
0.2	0	12.1 ± 8.9[b]
0.4	0	0

[a] Each value represents the mean ± SD of triplicate culture.
[b] $P < 0.05$ compared with infected cultures incubated without vincristine.

with G207 or vincristine alone. The combination of vincristine at concentrations of 0.1 and 0.2 ng/ml with G207 also strongly enhanced effects against KF-RMS-1 cells compared with a single treatment, whereas addition of 0.4 ng/ml vincristine to G207 resulted in death of all cells.

Vincristine Does not Influence Infection Efficiency and Viral Growth in Human Rhabdomyosarcoma Cells. To measure whether vincristine affects G207 infection/replication efficiency of human rhabdomyosarcoma cells, infected cells expressing *lacZ* were counted 4 days after G207 infection (MOI 0.1). G207 readily infected KFR cells, as demonstrated by LacZ-expression (*i.e.*, green-stained cells), and KF-RMS-1 cell cultures showed comparable infection levels (Table 2). Similar numbers of infected cells were found in cultures incubated with vincristine at concentrations ranging from 0.1 to 0.4 ng/ml (Table 2). Effects of vincristine on G207 replication in KFR and KF-RMS-1 cells were assessed by measurements of viral titers in cultures infected at MOI 0.1 4 days after infection. Input virus ranged from 1.2×10^2 to 2.8×10^2 PFU/ml. Addition of vincristine at concentrations ranging from 0.1 to 0.4 ng/ml did not significantly influence G207 replication in KFR and KF-RMS-1 cells (Table 2).

i.v. G207 Treatment of KFR and KF-RMS-1 Tumors. Growth of KFR as well as KF-RMS-1 tumors was significantly inhibited when treated with 2×10^7 PFU G207 i.v. on days 0 and 4 compared with control animals treated with virus buffer. KFR and KF-RMS-1 growth was inhibited 3.5- ($P < 0.001$) and 2.6-fold ($P < 0.001$) at day 22, respectively, compared with mock infection. Treatment with UV-B-inactivated G207 did not influence tumor growth (Fig. 2, *A* and *C*). Nevertheless, no complete tumor disappearance was observed in either treatment group (Table 3). Similar results were obtained from a second independent experiment (data not shown).

Intraneoplastic G207 Treatment of KF-RMS-1 and KFR Tumors. i.t. treatment with G207 alone (1×10^7 PFU in 20 μl on days 0 and 4) resulted in complete tumor disappearance in two of eight animals in the KF-RMS-1 group and two of eight animals in the KFR group at day 22 (Table 3). These animals were followed up for a period of 3 months after tumor cell inoculation. As no tumor regrowth was observed, they were defined to be cured. Moreover, treatment with i.t. G207 significantly inhibited tumor growth of the remaining KFR tumors (5.9-fold inhibition compared with mock-infected control tumors at day 22, $P < 0.001$; Fig. 2*B*) and KF-RMS-1 tumors (3.1-fold inhibition compared with mock infected control tumors at day 22, $P < 0.001$; Fig. 2*D*). Treatment with UV-B-inactivated G207 did not have any effect on tumor growth (Fig. 2, *B* and *D*). Similar results were obtained from a second independent experiment (data not shown).

Combined Treatment of KFR and KF-RMS-1 Tumors with i.v. G207 and Vincristine. Mice inoculated with KFR or KF-RMS-1 cells were treated with increasing concentrations of vincristine. The highest single dose of vincristine that did not lead to an increased mortality of mice was 0.5 mg/kg body weight in the absence of tumors (data not shown). Growth curves of tumors treated with 0.5 mg/kg body weight vincristine are shown in Fig. 3, *C* and *G*. Treatment significantly inhibited growth of both KFR (5.9-fold inhibition;

Table 2 *Effects of vincristine on infection efficiency and viral growth of G207 in KFR cells*

Treatment	% of infected cells (LacZ-positive cells)		Virus titre (PFU/ml)	
	KFR	KF-RMS-1	KFR	KF-RMS-1
G207 (MOI 0.1) + vincristine (ng/ml)				
0	75.6 ± 21.3[a]	69.5 ± 16.7	9.4×10^5	5.3×10^5
0.1	77.8 ± 11.4	66.7 ± 14.5	8.7×10^5	8.2×10^5
0.2	74.8 ± 10.2	65.1 ± 18.2	7.9×10^5	6.9×10^5
0.4	76.4 ± 16.9	68.2 ± 12.9	9.1×10^5	7.4×10^5

[a] Each value represents the mean ± SD of triplicate culture.

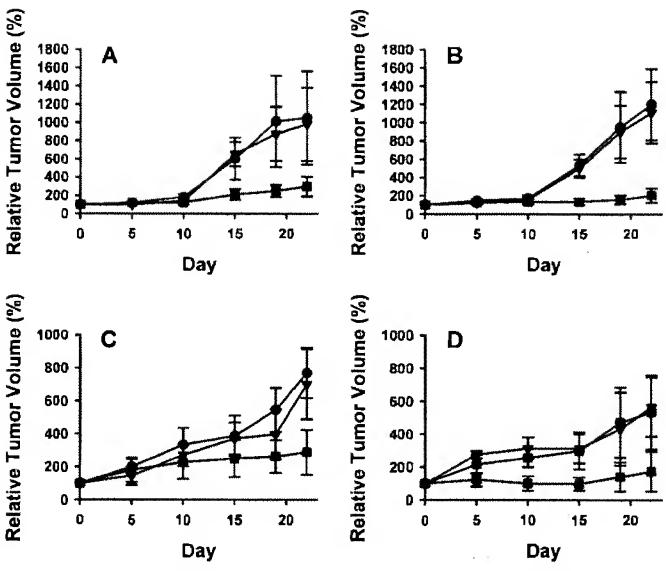

Fig. 2. Growth curves of rhabdomyosarcoma tumors in CD-1 (nu/nu) mice treated with G207 intraneoplastically or i.v. *A*, KFR tumors treated i.v. with G207 (2×10^7 PFU at days 0 and 4, ■), UV-B-inactivated G207 (2×10^7 PFU at days 0 and 4, ▼), or PBS (control, ●). *B*, KFR tumors treated intraneoplastically with G207 (1×10^7 PFU at days 0 and 4, ■) UV-B-inactivated G207 (2×10^7 PFU at days 0 and 4, ▼), or PBS (control, ●). Cures were noted in 25% of G207 intraneoplastically treated KFR tumor-bearing animals (two of eight animals). *C*, KF-RMS-1 tumors treated i.v. with G207 (2×10^7 PFU at days 0 and 4, ▼) UV-B-inactivated G207 (2×10^7 PFU at days 0 and 4, ■), or PBS (control, ●). *D*, KF-RMS-1 tumors treated intraneoplastically with G207 (1×10^7 PFU on days 0 and 4, ■), UV-B-inactivated G207 (1×10^7 PFU at days 0 and 4, ▼), or PBS (control, ●). Cures were noted in 25% of G207 intraneoplastically treated KF-RMS-1 tumor-bearing animals (two of eight animals). Data represents mean tumor growth ± SD.

$P = 0.002$, Fig. 3*C*) and KF-RMS-1 (1.6-fold inhibition; $P = 0.01$, Fig. 3*G*) xenografts compared with control animals at day 22.

Growth of both KFR and KF-RMS-1 tumors treated with G207 i.v. + vincristine was significantly inhibited compared with control animals at day 22. However, no cures were seen in animals carrying KF-RMS-1 (ERMS) tumors (2.6-fold growth inhibition compared with untreated control, $P < 0.001$; Fig. 3*H*, Table 3). In sharp contrast, in five of eight animals bearing KFR (ARMS), tumors (62.5%) were cured (*i.e.*, complete tumor regression with no tumor regrowth during a 3 month follow-up) by the combination treatment of G207 i.v. + vincristine (Table 3). In remaining KFR tumors, 7.4-fold growth inhibition ($P = 0.045$) compared with untreated control was observed at day 22 (Fig. 3*D*). Tumor growth inhibition between groups receiving single agent treatment did not differ significantly, whereas mean volumes of KFR and KF-RMS-1 tumors treated with G207 i.v. + vincristine were significantly smaller than tumors from animals treated with G207 i.v. ($P = 0.043$ for KFR; $P = 0.003$ for KF-RMS-1) or vincristine alone ($P = 0.049$ for KFR; $P = 0.005$ for KF-RMS-1; Fig. 3).

Detection of G207 Virus Infection in i.v. Treated KFR and KF-RMS-1 Tumors. The spreading of G207 infection in KFR and KF-RMS-1 tumors treated with G207 i.v. or with the combination of G207 i.v. + vincristine was examined by X-gal staining on days 1 and 5 of treatment. As shown representatively for KFR tumors, 1 day after the injection of virus only single, scattered cells expressing β-galactosidase were found in animals treated either with G207 or with the combination of G207 i.v. + vincristine (Fig. 4, *A* and *B*). Contrasting this, on day 5 of treatment both G207 i.v. and G207 i.v. + vincristine-treated tumors displayed similar extents of diffusely stained areas with numerous β-galactosidase-positive cells (Fig. 4, *C* and *D*). Single treatment with G207 i.v. of both rhabdomyosarcoma tumors and combined treatment of KF-RMS-1 tumors showed similar results (data not shown). The presence and replication of G207 in i.v. treated tumors were additionally confirmed by ultrastructural investigations. As representatively shown for KF-RMS-1, untreated controls revealed poorly differentiated rhabdomyoblasts with actin and myosin filaments and with interspersed glycogen particles (Fig. 5*A*). In tumors inoculated with the combination of G207 i.v. + vincristine or G207 i.v. as a single agent, numerous cells with different stages of HSV-1 morphogenesis consistent with viral productive infection (30) were observed at day 5 of treatment, *i.e.*, enveloped G207 HSV-1 particles in the cytoplasm (Fig. 5*B*), budding virus particles next to and through the plasma membrane (Fig. 5*C*), as well as viral nucleocapsids in the cell nucleus (Fig. 5*D*). These results demonstrate that therapeutic concentrations of vincristine did not prevent G207 infection and viral spreading in rhabdomyosarcoma cells *in vivo*.

DISCUSSION

In this study, we elaborated a novel approach for the treatment of rhabdomyosarcoma using multimutated oncolytic HSV-1 G207. G207 was an effective oncolytic agent *in vitro* for multiple rhabdomyosarcoma cell lines even when used at MOIs as low as 0.01. As clinical evidence indicates, a worse prognosis for patients with ARMS compared with patients with ERMS (5, 6), leading some clinicians to intensify therapy only for patients with unfavorable histology, *i.e.*, ARMS and not ERMS (39), we compared effects of G207 on the growth of four ERMS and four ARMS cell lines. All cell lines with the exception of ERMS cell line Rh1 were highly sensitive suggesting that G207 is an effective agent for the treatment of both rhabdomyosarcoma histological subtypes. Our results are extended by a recent report on high levels of oncolysis in two additional rhabdomyosarcoma cell lines infected with G207 at low MOIs, published during the preparation of the manuscript (40). Hence, we conclude that critical differences in genetic diversity underpinning the histological and biological diversity of ERMS and ARMS do not significantly modulate susceptibility to G207. Furthermore, our findings parallel studies with epithelial tumors demonstrating higher levels of oncolysis of G207 at lower MOIs than indicated in prior investigations against neuroectodermal tumor cell lines (41).

Both i.t. and i.v. treatment with G207 showed significant antitumoral activity against xenografts of human ERMS and ARMS cell lines. These results corroborate findings from other investigators that tumor cells susceptible to G207 *in vitro* at low MOI also show therapeutic susceptibility *in vivo*. G207 showed strong antitumoral activity when injected twice at a PFU of 1×10^7 i.t. at days 0 and 4 inducing complete disappearance of tumors in about a quarter of both KFR- and KF-RMS-1-bearing animals (Table 3). As we did not find tumor regrowth during 3 months follow-up period, we defined these animals to be cured.

Table 3 *Intratumoral and systemic i.v. treatment of KFR and KF-RMS-1 tumors with G207, vincristine, UV-B-irradiated G207 and combination of G207 and vincristine*

	No. of cured animals[a]/no. of treated animals				
	Control	G207	Vincristine	UV-B-irradiated G207	G207 + vincristine
KFR tumors					
i.t. treatment	0/8	2/8	n.d.[b]	0/8	n.d.
Systemic (i.v.) treatment	0/8	0/8	0/8	0/8	5/8
KF-RMS-1 tumors					
i.t. treatment	0/8	2/8	n.d.	0/8	n.d.
Systemic (i.v.) treatment	0/8	0/8	0/8	0/8	0/8

[a] Cures were defined as total tumor regressions without regrowth during a 3 month follow-up period.
[b] n.d., not done.

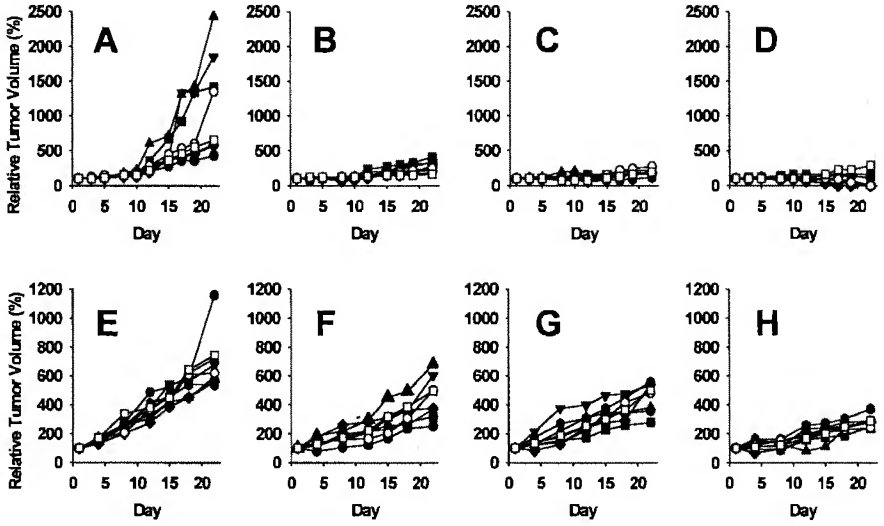

Fig. 3. Growth curves of alveolar rhabdomyosarcoma KFR (*A–D*) and embryonal rhabdomyosarcoma KF-RMS-1 (*E–H*) tumors in CD-1 (nu/nu) mice. *A* and *E*, tumor-bearing mice treated with PBS (control); *B* and *F*, tumor-bearing mice treated with G207 (2×10^7 PFU i.v. on days 0 and 4); *C* and *G*, tumor-bearing mice treated with vincristine (0.5 mg/kg body weight on day 0); *D* and *H*, tumor-bearing mice treated with combination of G207 (2×10^7 PFU i.v. on days 0 and 4) and vincristine (0.5 mg/kg body weight on day 0). In the alveolar RMS KFR group treated with the combination of G207 and vincristine, five of eight animals (62.5%) were cured (*D*).

Systemic i.v. administration of oncolytic viruses, including adenovirus (42), vaccinia virus (43), and reovirus (44) into immune-compromised animals bearing different tumors was found to be effective, with evidence of active virus replication in tumor cells remote from the virus injection site. Previously, G207 administration by i.v. injection had a significant antitumor effect on s.c. xenografts of human prostate carcinoma (21) and intravesicular bladder carcinoma (45), as well as lung metastatic breast cancer (46). In this study, we showed the importance of active viral replication for the antitumoral effect of i.v. administered G207 to human rhabdomyosarcoma xenografts by demonstrating an increase of infected tumor cells (staining positively with X-gal) over time and productive viral infection in tumor cells by electron microscopy. These findings raise the possibility of systemic i.v. therapy with G207 for patients with metastatic rhabdomyosarcoma by approaches such as combination of G207 with immune suppressants, which were found not to abolish oncolytic activity of the virus (27).

On the other hand, HSV-1 was shown to induce host-innate immune responses consisting of activated macrophages, cytotoxic natural killer cells, and cytokines independently of virus replication (47), which may result in elimination of virus-infected tumor cells. To evaluate the importance of such viral replication-independent effects, KFR and KF-RMS-1 tumors were treated both i.t. and i.v. with UV-irradiated G207. We showed that G207 exerted antitumoral effects mainly because of viral replication as only infectious but not inactivated virus suppressed growth of both KFR and KF-RMS-1 tumors. These observations suggest that an innate immune response may not play a major role in the antitumoral activity of G207 on both KFR and KF-RMS-1 tumors. These results are consistent with previous observations using oncolytic HSV-1 for the treatment of murine tumors (48, 49). In these studies, oncolytic HSV-1 (G207 or 1716) did not exert antitumoral activity when used for syngeneic murine tumors in athymic nude, SCID, or leukopenic mice lacking CD4 and CD8 lymphocyte activity. In contrast, growth of corresponding tumors was inhibited when treated in immune-competent mice because of the activity of CD4 and CD8 lymphocytes but not of cells of the innate immune system (48, 49). In addition, heat-inactivated G207 did not exert antitumoral activity on human colon carcinoma xenografted tumors when compared with replication-competent G207 (22).

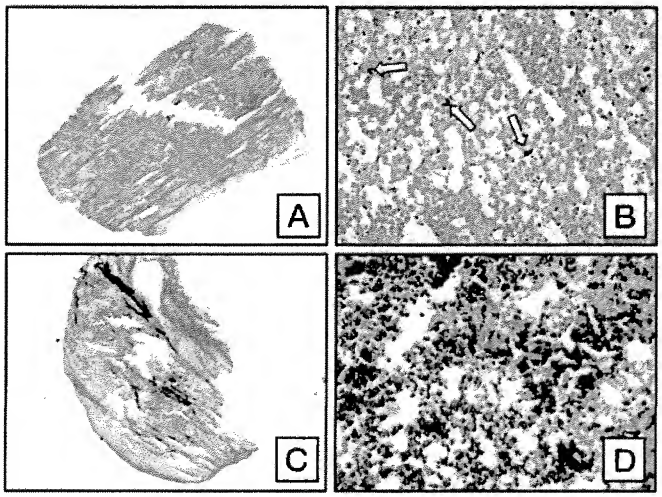

Fig. 4. s.c. xenografts of KFR tumors treated with i.v. G207 + vincristine, fixed on day 1 (*A* and *B*) and on day 5 (*C* and *D*). Samples were fixed with ethanol, stained for β-galactosidase (*blue*), and then counterstained with eosin. Macroscopic view (*A* and *C*) and microscopic magnification (*B* and *D*).

Because conditionally replicating HSV-1 vectors such as G207 kill tumor cells through pathways that are different from other anticancer therapies, it is reasonable to explore their interactions with commonly used antineoplastic agents. This combination may be superior to a combination of two cytostatic drugs because of absence of additive side effects of anticancer drugs. In this study, we tested whether vincristine, which is commonly used for the treatment of rhabdomyosarcoma, increased efficacy of G207 against murine xenograft models of ARMS using the KFR cell line and ERMS using KF-RMS-1 cells. The results showed that vincristine at effective concentrations did not influence G207 replication *in vitro*, whereas the combination of G207 + vincristine strongly increased cell killing of both cell lines when compared with treatment with either single agent. A similar observation has been made by Toyoizumi *et al.* (28) showing increased cell killing of human non-small cell lung cancer cell line treated by the combination of oncolytic HSV-1 1716 with mitomycin C without increase in viral replication. The recent results are especially of note considering that antimicrotubule agents such as vincristine may interfere with HSV infection (29). Moreover, combination of i.v. injected G207 with a single dose of vincristine induced cures in five of eight animals harboring alveolar KFR tumors, whereas single treatments inhibited tumor growth but led to no cures. Contrasting this, treatment of animals bearing KF-RMS-1 tumors with the com-

Fig. 5. Detection of G207 in KF-RMS-1 tumor-bearing CD-1 (nu/nu) mice treated with the combination of i.v. G207 (2×10^7 PFU) + vincristine (0.5 mg/kg) by electron microscopy. Ultrastructural investigations were performed on day 5 of treatment. Untreated control tumors revealed poorly differentiated rhabdomyoblasts (*A*). In mice treated with G207 + vincristine, cells with enveloped viral particles in the cytoplasm (*arrow*) were observed (*B*). Some infected cells showed budding G207 virus particles next to (*arrowhead*) and through (*arrow*) the plasma membrane (*C*); overview of part of the nucleus of infected cell with viral nuclepcapsids (*arrows*), which are shown in greater magnification in the *inset* (*D*); *n* = nucleus, c = cytoplasm.

bination of i.v. G207 and vincristine did not induce any cures, resulting, however, in significantly greater antitumoral effect when compared with treatment with either single agent. The differences of KFR and KF-RMS-1 cells in susceptibility to G207-mediated oncolysis, which was more obvious in the *in vivo* experiments than *in vitro*, may be because of multiple factors encountered under *in vivo* and *in vitro* conditions. For example, KF-RMS-1 tumor growth in nude mice was slower than that of KFR tumors, suggesting that the slower growing cells do not permit G207 replication as efficiently as more rapidly growing cells. This assumption is additionally supported by the finding that antitumoral effects of single vincristine, which may also depend on tumor growth rate, was lower in animals bearing slower growing KF-RMS-1 tumors than in animals carrying KFR tumors, *i.e.,* 1.6- and 5.9-fold growth reduction, respectively. Our results are corroborated by studies comparing the susceptibility of various human colon carcinoma cell lines to G207. These showed an association of sensitivity with doubling time, S-phase fraction, and growth rate *in vivo* (22). Other explanations may be that the two xenografted cell lines differ in their *in vivo* enzyme activity of RR and GADD 34 compensating the deleted genes of the HSV-1 G207 genome, *i.e.,* the *ICP6* and *γ34.5* gene, respectively, that critically determine rate of viral replication and host cell apoptotic behavior of G207-infected cells (50, 51).

In conclusion, we were able to show that i.t. and i.v. delivery of G207 significantly inhibited human rhabdomyosarcoma from both histological types, hence providing background for future clinical evaluation in patients suffering from metastatic rhabdomyosarcoma disease. Furthermore, the combination of G207 with vincristine, a well-known agent for rhabdomyosarcoma therapy, demonstrated highly enhanced antineoplastic activity in preclinical models of both ERMS and ARMS. Although in this study we used a single dose of vincristine, it will be of interest to determine whether a combination of G207 with vincristine or other antineoplastic drugs would be efficacious as "high time therapy," a novel cancer therapy concept based on nontoxic frequent low dosing of cytotoxic agents. This treatment may result in tumor elimination by suppression of angiogenesis rather than direct tumor cell killing (52, 53). It is conceivable that conditionally replicating HSV vectors could improve such a therapeutic approach because of their high selectivity for tumor tissues, as well as potential to destroy drug resistant tumor cells (23, 54). To summarize, these results encourage further evaluation of oncolytic G207 therapy for patients with high-risk rhabdomyosarcoma.

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